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Detection of the plasmid-mediated colistin-resistance gene *mcr-1* in faecal metagenomes of Dutch travellers

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Background: Recently, the first plasmid-mediated colistin-resistance gene, *mcr-1*, was reported. Colistin is increasingly used as an antibiotic of last resort for the treatment of infections caused by carbapenem-resistant bacteria, which have been rapidly disseminating worldwide in recent years.

Objectives: The reported carriage rate of *mcr-1* in humans remains sporadic thus far, except for those reported in Chinese populations. We aimed to determine its presence in the faecal metagenomes of healthy Dutch travellers between 2010 and 2012.

Methods: Faecal metagenomic DNA of pre- and post-travel samples from 122 healthy Dutch long-distance travellers was screened for the presence of *mcr-1* using a TaqMan quantitative PCR assay, which was designed in this study. All positive samples were confirmed by sequencing of the amplicons.

Results: The *mcr-1* gene was detected in 6 (4.9%, 95% CI = 2.1%–10.5%) of 122 healthy Dutch long-distance travellers after they had visited destinations in South(-east) Asia or southern Africa between 2011 and 2012. One of these participants was already found to be positive before travel.

Conclusions: Our study highlights the potential of PCR-based targeted metagenomics as an unbiased and sensitive method to screen for the carriage of the *mcr-1* gene and suggests that *mcr-1* is widespread in various parts of the world. The observation that one participant was found to be positive before travel suggests that *mcr-1* may already have disseminated to the microbiomes of Dutch residents at a low prevalence, warranting a more extensive investigation of its prevalence in the general population and possible sources.

Introduction

Colistin is increasingly used as an antibiotic of last resort for the treatment of infections caused by carbapenem-resistant bacteria. Therefore, the finding of plasmid-mediated colistin resistance has rightfully drawn renewed attention to colistin resistance.[1,2] Considering the successful dissemination of other resistance mechanisms by horizontal gene transfer,[3] the mobile colistin-resistance gene *mcr-1* may have major implications for public health. After the initial report by Liu *et al.*[4] describing a high prevalence of *mcr-1* in *Escherichia coli* isolates from pigs at slaughter (21%) and a relatively lower prevalence in *E. coli* and *Klebsiella pneumoniae* isolates from human patients (1.4% and 0.7%, respectively), more studies have been conducted investigating the worldwide spread of this gene.[2,5–12] While the rates of *mcr-1*-positive strains isolated from animals or food were quite high in some of these studies,[11,12] the reported carriage rate in humans thus far remains sporadic except for those reported in Chinese populations.[2] In contrast to most studies to date, which have mostly screened existing collections of cultured isolates or mined metagenomic sequencing data, we applied a PCR-based targeted metagenomic approach to detect the presence of the *mcr-1* gene in the faecal metagenomes of healthy Dutch travellers between 2010 and 2012.

Materials and methods

In this study we used faecal samples from 122 healthy Dutch long-distance travellers previously recruited in a prospective travel cohort study.[13] Processing and DNA extraction of faecal samples was described

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previously.[13] To use a targeted metagenomics approach, a TaqMan quantitative PCR (qPCR) assay was developed. In addition to the primers described by Liu *et al.*,[4] generating a 309 bp product, a dual-labelled probe was designed [5′-TTGACCGCGACCGCCAATCTTA-3′ with FAM reporter and a Black Hole Quencher (BHQ1)] (Sigma-Aldrich, St Louis, MO, USA). Amplifications were performed on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in 25 μL reactions containing 12.5 μL of ABsolute QPCR ROX Mix (Thermo Fisher Scientific), 5 μL of template DNA, 300 nM of both primers and 200 nM of probe. Thermal cycling consisted of 15 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. To assess the efficiency of the assay, standard curves were created using a control plasmid. This plasmid was constructed by cloning the corresponding PCR amplicon into a pGEM-T easy vector (Promega Corporation, Madison, WI, USA). Standard curves were then generated by triplicate measurements of serial dilutions of the control plasmid spiked into three different (faecal) metagenomic DNA samples.

All faecal samples indicated as positive by qPCR were confirmed by sequencing using the PCR primers and a BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific) and were analysed using NCBI's Basic Local Alignment Search Tool (BLAST).

Faecal samples that tested positive for the *mcr-1* gene were streaked on to lysogenic broth agar plates containing colistin (2 mg/L) and vancomycin (50 mg/L) and incubated overnight at 37°C to obtain colistin-resistant isolates. Once the cultures were visually confirmed to be pure and resistant to colistin, they were streaked on to blood agar plates (Becton-Dickinson, Franklin Lakes, NJ, USA) for identification by MALDI-TOF MS (bioMérieux, Marcy-l'Étoile, France) and antibiotic susceptibility testing using the BD Phoenix Automated Microbiology System (Becton-Dickinson) and Etest for colistin (bioMérieux); results were interpreted according to EUCAST.[14]

PFGE was performed on colistin-resistant *E. coli* isolates according to the rapid procedure described previously.[15] After 15 min of pre-incubation in 200 μL of the restriction enzyme buffer, supplemented with 100 μg/mL BSA, ∼2×5 mm thick slices of each plug were incubated at 37°C for 3 h with 30 U of XbaI enzyme in 100 μL of the restriction enzyme buffer + 100 μg/mL BSA. Fragments were separated on a 1% SeaKem Gold agarose gel for 16 h. *Salmonella* serotype Braenderup H9812 strain (ATCC strain BAA-664) was included as a reference strain. The resulting gel was analysed with BioNumerics v7.5 and according to the criteria of Tenover *et al.*[16] MLST was performed according to Wirth *et al.*[17] Real-time PCR to detect bla_{CTX-M}, *qnrA*, *qnrB* and *qnrS* genes in *mcr-1*-harbouring isolates obtained in this study was performed as described previously.[13]

Results

The designed *mcr-1* assay was linear over 8 log dilutions of a control plasmid (C_t value range = 10.7–33.5, maximum variation between replicates = 0.42 C_t, slope = −3.378, R^2 = 0.999), with an efficiency of 98%. While concentrations of ∼5 plasmid copies per reaction were no longer within the linear reach and showed greater variation between replicates (C_t value = 36.4–37.7), they were none the less reproducibly detectable. Measuring log dilutions of the control plasmid spiked into DNA isolated from faecal samples yielded a similar efficiency (slope = −3.341, R^2 = 0.999) and sensitivity.

Screening of the faecal metagenomes of 122 travellers, before and after travel, yielded seven samples positive for *mcr-1* according to qPCR (C_t value range = 24.1–40.2). Positive samples were all confirmed by sequencing, showing high specificity of the PCR assay (no false-positives were observed). For one participant, the *mcr-1* gene was detected in both the pre- and post-travel sample, whereas for five other participants only the post-travel sample was found to be positive, reflecting a post-travel prevalence of 4.9% (95% CI = 2.1%–10.5%) and acquisition rate of 4.1% (95% CI = 1.5%–9.6%) (Table 1). Half of the participants who were positive for *mcr-1* after travel had used antibiotics in the 3 months preceding post-travel sampling (Table 1) as compared with only 9.5% of participants negative for *mcr-1* ($P < 0.05$).

The seven faecal samples of participants that tested positive for *mcr-1* with the targeted metagenomic approach were cultured to obtain colistin-resistant Gram-negative bacteria. Three *mcr-1*-harbouring, colistin-resistant *E. coli* isolates were obtained, which are specified in Table 2. Although two of these isolates were obtained from participant 6 [one before travel (isolate 6T0) and one after travel (isolate 6T1)], these strains were

Table 1. Characteristics of participants positive for *mcr-1* carriage and antibiotic-resistance genes detected in faecal metagenomes of 122 Dutch international travellers

Participant no.	Age (years)	Sex	Travel duration (days)	Antibiotic used in the 3 months preceding post-travel sampling	Destination(s)	Date of return	Antibiotic-resistance genes in faecal metagenome pre-travel	Antibiotic-resistance genes in faecal metagenome post-travel
1	55	male	5	minocycline	Thailand	May 2011	—	*mcr-1*, *qnrS*
2	69	male	21	—	Vietnam	March 2011	*qnrB*	*mcr-1*, CTX-M-2[a], CTX-M-9[a], *qnrA*, *qnrB*, *qnrS*
3	24	female	18	—	Indonesia	June 2011	—	*mcr-1*, *qnrS*
4	26	female	21	—	Tanzania	June 2011	—	*mcr-1*, *qnrS*
5	40	female	35	trimethoprim/sulfamethoxazole	Thailand and Vietnam	January 2012	—	*mcr-1*, *qnrB*, *qnrS*
6	46	female	10	azithromycin	India	May 2012	*mcr-1*, *qnrB*	*mcr-1*, *qnrB*, *qnrS*

[a]CTX-M phylogenetic group as determined by qPCR.

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Table 2. MLST and antibiotic susceptibility testing profiles of *mcr-1*-harbouring *E. coli* isolated from Dutch human faecal samples

	Isolate ID (MLST)					
	2T1 (ST 1011)		6T0 (ST 744)		6T1 (ST 80)	
	MIC (mg/L)	S/R[a]	MIC (mg/L)	S/R	MIC (mg/L)	S/R
Amikacin	≤4	S	≤3	S	≤4	S
Amoxicillin/clavulanate	**32/2**	**R**	4/2	S	4/2	S
Ampicillin	**>8**	**R**	**>8**	**R**	≤2	S
Cefepime	≤1	S	≤1	S	≤1	S
Cefoxitin	≤4	S	≤4	S	≤4	S
Ceftazidime	≤1	S	≤1	S	≤1	S
Ceftriaxone	≤1	S	≤1	S	≤1	S
Cefuroxime	4	S	4	S	≤2	S
Ciprofloxacin	**>1**	**R**	**>1**	**R**	≤0.25	S
Colistin[b]	**3**	**R**	**3**	**R**	**3**	**R**
Ertapenem	≤0.25	S	≤0.25	S	≤0.25	S
Gentamicin	≤1	S	≤1	S	≤1	S
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S
Levofloxacin	**>2**	**R**	**>2**	**R**	≤0.5	S
Meropenem	≤0.125	S	≤0.125	S	≤0.25	S
Piperacillin	**>16**	**R**	**>16**	**R**	≤4	S
Piperacillin/tazobactam	≤4/4	S	≤4/4	S	≤4/4	S
Temocillin	16	—	≤4	—	≤4	—
Tigecycline	≤0.5	S	≤0.5	S	≤0.5	S
Tobramycin	2	S	≤1	S	≤1	S
Trimethoprim/sulfamethoxazole	**>4/76**	**R**	**>4/76**	**R**	≤1/19	S

R, resistant; S, susceptible; —, no breakpoint available.
Bold formatting indicates resistance.
[a]Interpretation according to EUCAST clinical breakpoints.
[b]As determined by Etest.

distinct as determined by PFGE (data not shown) and MLST (Table 2) and showed different antibiotic susceptibility testing profiles (Table 2). Considering the acquisition of other resistance genes in the faecal microbiomes mentioned in Table 1, the isolated *mcr-1*-positive strains were also tested for the presence of $bla_{CTX\text{-}M}$, *qnrA*, *qnrB* and *qnrS* genes, but were negative.

Discussion

In our study, we report the occurrence of *mcr-1* in the faecal microbiota of Dutch international travellers using a targeted, PCR-based metagenomic approach. Bontron *et al*.[18] previously described a SYBR Green-based real-time PCR to overcome false-positive signals, which were observed with earlier published primers. However, since metagenomic DNA samples are more prone to induce unspecific amplification due to the diversity of DNA templates present compared with, for example, DNA isolated from bacterial isolates, we opted for the addition of a TaqMan probe in our assay. The designed TaqMan assay was shown to be both specific and sensitive when used on isolated as well as on metagenomic samples.

Bacteria harbouring *mcr-1* could be cultured from some, but not all, investigated faecal samples that tested positive for this gene by qPCR. Likely, the (viable) bacterial load in the samples was too low or was negatively affected by storage, as there was also an absence of growth of any other colistin-resistant Gram-negative bacteria. Alternatively, the *mcr-1* genes could be non-functional, non-expressed or originate from organisms not readily cultivable with the currently used methods.

This study shows a post-travel prevalence (4.9%) of *mcr-1* in the microbiota of travellers. In line with a recent other Dutch study,[19] the pre-travel prevalence in the present study was much lower (0.8%). The observed acquisition rate suggests, however, that *mcr-1* has already disseminated at high rates in certain parts of the world and travel to such high-risk areas is a risk factor for its acquisition and further dissemination. Concerning this, Arcilla *et al*.[20] previously reported that 6 (<1%) of 633 ESBL-producing Enterobacteriaceae that were acquired by Dutch international travellers were positive for *mcr-1*. In our study, we did not select for travel-associated ESBL-producing isolates, but rather screened all faecal metagenomes, which most likely accounts for the higher acquisition rate.

Many studies on *mcr-1* have investigated collections of ESBL-producing Enterobacteriaceae and it has been reported that *mcr-1*-positive isolates often carry multiple resistance genes, including genes encoding ESBLs.[2] For this reason, we investigated the antibiotic resistance profiles of the three isolates obtained from our study population. These were all determined to be

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non-ESBL producers, however. Notably, one isolate (6T1) was susceptible to all tested antibiotics other than colistin. To this end, there is a need for studies without selections based on resistance profiles to define this relationship more clearly.

In conclusion, we report a relatively high *mcr-1* prevalence in microbiomes of Dutch residents after international travel, highlighting the potential of PCR-based targeted metagenomics as an unbiased and sensitive method to screen for the carriage of *mcr-1*. The acquisition rates show that travel contributes to the dissemination of *mcr-1*. Furthermore, the participant who was *mcr-1*-positive before travel indicates that *mcr-1* may already be present in the microbiomes of Dutch residents at a low prevalence, warranting further investigation of its prevalence in the general population and possible sources.

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Transparency declarations

None to declare.

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